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Deregulation of the activity of the serine/threonine kinase Akt is likely to play an important role in initiation and/or progression of breast carcinomas. The goal of our studies is to characterize the effector-mediated pathways used by Akt to promote cell survival and to trigger malignant transformation. We have used a yeast two-hybrid approach to identify proteins that interact with Akt. C21, one of the isolates recovered in the screen, interacts with Akt in vitro and is a substrate for the Akt kinase. C21 is a transcription factor. Thus, Akt may trigger malignant transformation and promote cell survival in part by altering gene expression.

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Anne B. Vojtek 5/25/99

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Introduction

Akt is a serine/threonine kinase that is involved in the initiation and/or progression of breast carcinomas. The cellular and molecular events targeted by Akt to promote cancer are not well documented. Akt could induce malignant transformation by constitutively activating a growth signaling pathway by serine/threonine phosphorylation of key intermediates in this pathway. Alternatively, Akt could prevent apoptosis (programmed cell death) by serine/threonine phosphorylation of key components of an apoptosis pathway. The key to understanding how Akt promotes malignant transformation is to identify its cellular targets.

Work Accomplished To Date

We proposed to identify effectors and regulators of Akt (Task 1, months 1-18). We proposed 7 steps for the completion of Task 1 and have currently completed 4 of 7. Experiments directed towards the completion of the remaining three steps of Task 1 are in progress.

Identification of Akt interacting proteins

We proposed to identify targets of Akt using a yeast two-hybrid screen (1). We have successfully completed this screen, Figure 1 (Task 1, part a and b). 5 million yeast transformants were screened (approximately one times the complexity of the mouse embryo library). We recovered 16 clones that specifically interacted with Akt: a positive interaction in the two-hybrid system was observed between the 16 clones and LexA-Akt but not LexA-Lamin. LexA-Lamin is a control fusion to the LexA DNA binding domain, which is commonly used to reveal false positives in two-hybrid screens. The library clones were recovered from yeast and transformed into E. coli. The library clones were placed into six classes by restriction mapping of the library inserts with Sau3A1 and examples of each class were sequenced (Task 1, part c). These six classes comprise several known proteins as well as several uncharacterized proteins present in the data base only as expressed sequence tags (ESTs). At the present time, we have focused on one of our classes, C21.

C21 encodes the carboxyl terminal 123 amino acids of Brn1, a POU domain transcription factor. We have focused on Brn1 for three reasons. First, Brn1 contains an amino acid sequence that matches the consensus site for phosphorylation by Akt and, as discussed below, we have demonstrated that Akt phosphorylates Brn1 in vitro. Second, the likely phosphorylation site is conserved in all members of this family of transcription factors, suggesting a general role for Akt in their biology. Third, Brn3a, which is highly related to Brn1 (35% conservation in sequence similarity between the two in the region identified in the two hybrid screen) promotes both cell survival and transformation.

POU domain transcription factors are critical regulators of key developmental processes (reviewed in 2). Many POU domain proteins are expressed in the developing nervous system, where they are believed to function in early embryogenesis as well as during the terminal differentiation of specific neuronal lineages. Targeted deletion of the genes of individual members of this family in mice suggests that these transcription factors also regulate neuronal survival. In

addition, a recent study has demonstrated that the Brn-3a POU domain transcription factor protects sensory neurons from programmed cell death induced by withdrawal of nerve growth factor (3). This study also showed that Brn-3a is capable of activating the *bcl-2* gene promoter, resulting in enhanced Bcl-2 protein levels. Thus, Akt may exert its survival-promoting effects in part by activating Brn-3a, thereby upregulating the levels of Bcl-2 and/or other survival factors. Since the likely site of phosphorylation of Brn-3a by Akt is conserved in all members of the POU domain family of transcription factors, the effects of Akt on survival may be mediated by multiple members of this family. Moreover, Brn-3a has been reported to transform mammalian cells in cooperation with Ras (4). Thus, Akt may both promote malignant transformation and induce cell survival through alteration of the regulation of POU family members.

In a recent study, four members of the POU domain family were found to be expressed in breast cancer cell lines, Oct1, Oct2, Oct3 and Oct11; one of these, Oct3, is expressed in human primary breast carcinomas but not in normal human breast cancer lines (5). Therefore, our studies in the future will also focus on the regulation of Oct3 by Akt.

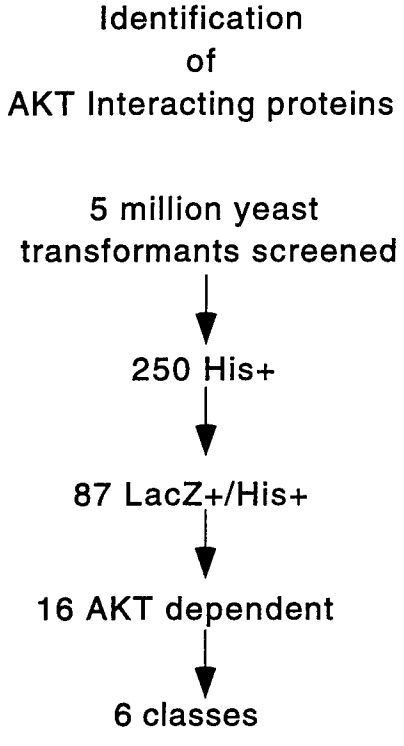

Figure 1. Summary of library screen. 1:312,500 yeast transformants screened exhibited Akt-dependent transactivation of both the LacZ and His3 reporter constructs. Restriction mapping and sequencing revealed that these 16 isolates represent 6 classes.

Akt and Brn1 interact in vitro (Task1, part d)

To confirm our yeast two-hybrid experiments and to provide secondary evidence for a physical association between Akt and the Brn1 isolate, we assessed the interaction between Akt and Brn1 in vitro. Human embryonic kidney 293 cells were transiently transfected with expression vectors encoding glutathione-S-transferase (GST) or Akt tagged at its amino terminus with GST (GST-AKT). Forty-eight hours after transfection, cells were harvested and extracts prepared. The GST and GST-AKT fusion proteins were isolated from the cell extracts by the addition of glutathione-sepharose. The Brn1 isolate was subcloned into pCS3+MT and the protein was prepared by in vitro transcription/translation in reticulocyte lysate in the presence of [35-S]-methionine. Equal quantities of GST or GST-AKT bound to sepharose were incubated with [35-S]-methionine labeled Brn1, the resin was washed, and then the presence or absence of Brn1 detected by autoradiography after SDS-PAGE. We observe binding of Brn1 to GST-AKT but not GST, Figure 2. Thus, Brn1 and Akt can interact in vitro. To determine if this interaction is direct or bridged or modulated by additional proteins, the interaction will be assessed in vitro using proteins purified from bacteria.

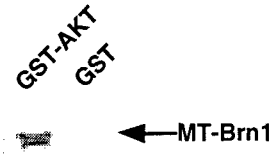

Figure 2. Akt associates with Brn1 in vitro. Purified GST or GST-AKT bound to glutathione-sepharose was incubated with [35-S]-methionine labeled MT-Brn1. The resin was washed and the presence or absence of MT-Brn1 assessed by SDS-PAGE followed by autoradiography. MT-Brn1 associates with GST-AKT but not GST. MT alone does not bind GST or GST-AKT (not shown).

Akt phosphorylates Brn1 in vitro (Task 1, part e)

The Brn1 isolate we recovered in the two-hybrid screen contains the amino acid sequence RKKRTSI, which matches the consensus Akt phosphorylation motif RXRXXS(hydrophobic) found in known Akt substrates GSK3 and Bad. To determine whether Brn1 is an Akt substrate, in vitro kinase assays were performed. Human embryonic kidney 293 cells were transiently transfected with expression vectors for GST or GST-AKT. The cells were grown to confluence, serum starved, and then stimulated with insulin to activate Akt catalytic activity. Cells were also pretreated or not with LY294002 prior to insulin stimulation. LY294002 is a specific inhibitor of phosphatidylinositol 3-kinase enzymatic activity. Akt activity is dependent on the lipid products produced by PI3K and, therefore, inhibiting the enzymatic activity of PI3K by LY294002 thus inhibits the activation of Akt. After

stimulation with insulin, extracts were prepared and active GST-AKT or inactive GST-AKT (from cells pretreated with LY294002) was collected on glutathione-Sepharose. Brn1 was epitope tagged with maltose binding protein (MBP) and purified from bacteria. Purified MBP-Brn1 or MBP was incubated with active or inactive GST-AKT in the presence of γ-[32-P]- ATP. The incorporation of γ-[32-P]-ATP into substrate was detected by SDS-PAGE of the kinase reactions followed by autoradiography. GST-AKT phosphorylated MBP-Brn1 but not MBP and the phosphorylation was reduced when the GST-AKT was harvested from cells pretreated with LY294002, Figure 3. Thus, Akt phosphorylates Brn1 in vitro. In order to determine the site of phosphorylation, we are constructing mutants in the RKKRTSI sequence. T and S will be altered to alanine each alone, and in combination, to create potentially non-phosphorylatable versions of Brn1.

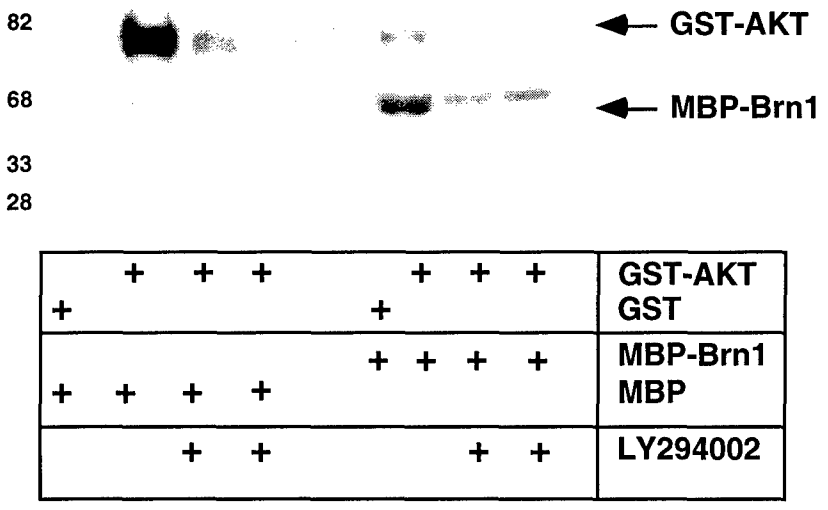

Figure 3. Akt phosphorylates Brn1 in vitro. Gst or GST-AKT, harvested from cells stimulated with insulin and treated or not with LY294002 (50 uM lanes 3 , 7; 100 uM lanes 4, 8), was incubated with MBP or MBP-Brn1 in the presence of γ-[32-P]-ATP. The kinase reaction was analyzed by SDS-PAGE followed by autoradiography. GST-AKT phosphorylates MBP-Brn1 but not MBP. Phosphorylation of MBP-Brn1 is not seen when GST-AKT is inactive (harvested from cells pretreated with LY294002). Upper arrow shows the position of the autophosphorylated GST-AKT.

Future Directions

Our work to date suggests that Akt will both promote malignant transformation and induce cell survival through alteration of the regulation of POU family members. Our efforts in the coming year will be directed towards completing Task 1 and making or acquiring the reagents needed for Task 2. To begin Task 2, we need to obtain full length clones of the POU family members, Brn1 and Brn3a. In addition, we will also make or acquire a full length clone of Oct3, since this POU protein is expressed in human primary breast carcinomas but not normal breast epithelial cells. We have requested full length clones by mail. If these requests are not met, then we will clone full length versions of the POU family members by screening cDNA or genomic libraries or by RT-PCR strategies (Task 1, parts f and g). In addition, once we have identified the sites phosphorylated by Akt we will alter the phosphorylation site(s) to alanine to create non-phosphorylatable versions of the substrates (putative dominant negative mutants) and to aspartic acid to create a constitutively phosphorylated/activated version of the substrate (putative dominant positive). These mutants will be quite useful for our transformation and cell survival studies in Task 2.

Key Research Accomplishments

- Identified Akt interacting proteins using a yeast two-hybrid screen
- Provided secondary evidence of complex formation between Akt and an Akt interacting protein, Brn1
- Demonstrated that Brn1 is an in vitro substrate for the Akt kinase

Reportable Outcomes

- Dr. Teresa Brtva, a postdoctoral fellow, successfully obtained salary support upon funding of an NIH NRSA (the DOD grant continues to provide supplies for Dr. Brtva's work)
- This award also supports the stipend and tuition of Claudia Figueroa, a graduate student in the Department of Biological Chemistry

Conclusions

Akt is a serine/threonine kinase that is involved in the initiation and/or progression of breast carcinomas. The cellular and molecular events targeted by Akt to promote cancer are not well documented. The key to understanding how Akt promotes malignant transformation is to identify its cellular targets. Using a yeast two-hybrid approach, we have identified likely targets for Akt, including a member of the POU domain family of transcription factors. Our work to date suggests that Akt-mediated regulation of POU domain transcription factors may be one mechanism by which Akt promotes cell survival and transformation.

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